STUDIES OF THE EFFECT OF IRRADIATION OF CELLULAR PARTICULATES. VI. COMPARISON OF UNCOUPLING AT THE THREE PHOSPHORYLATING SITES[1]

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It is well established that exposure of rats to whole-body irradiation results in the uncoupling of oxidative phosphorylation. Mitochondria isolated from various organs show a decreased P:O ratio, so long as the assay is performed at least eight hours after irradiation (Potter and Bethel, 1952; van Bekkum, 1957; Yost, Glickman and Beck, 1964). The majority of this work has been done by measuring uncoupling at the level of the oxidation of cytochrome-*b* (succinate substrate), while a few workers have worked with the initial step, primarily using glutamate as a substrate to reduce pyridine (Hall, Goldstein and Sonnenblick, 1963; Goldstein and Hall, 1965). Recently, we have demonstrated uncoupling at the terminal step, the oxidation of cytochrome-*c* reduced by ascorbate (Yost, Robson and Yost, 1967). These last results indicated that the terminal step in the phosphorylation chain is the most sensitive to uncoupling by ionizing radiations, and this, in turn, suggested the possibility that all of the observed uncoupling at other sites might be merely a reflection of damage done to the terminal step.

There are a number of possibilities for the inactivation of the phosphorylating mechanism. To cite the most obvious, it is possible that all of the uncoupling occurs in the terminal step of the chain. If this were the case, the highest percentage inactivations would be observed when using ascorbate as a substrate; the inactivation at the second step (succinate substrate) should be approximately one-half that observed with the ascorbate; and the inactivation observed in the primary step (glutamate substrate) would be only one-third that observed with ascorbate. If, on the other hand, a higher percentage inactivation were observed in the initial or second step, such an hypothesis would be untenable. Therefore, it seemed worthwhile to make comparative studies of the uncoupling of all three sites to determine whether the inactivation occurs at that one common to all three pathways.

Unfortunately, we cannot use the results obtained previously by various investigators to make the necessary comparisons. The variations in technique are sufficient to cause a rather wide variation in results. In fact, it is difficult to take results from any one laboratory, obtained at different times, and compare them (Yost, Robson and Yost, 1967). Thus, it seemed wise to observe the uncoupling of all three steps in one laboratory, under a single set of conditions, so that comparative studies of the effect of uncoupling on the three different phosphorylating sites could be made.

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Materials and Methods

All experiments were carried out with male, albino rats of the Sprague-Dawley (Gofmoor Farms) and CD (Charles River Sprague-Dawley) strain, weighing between 170 and 210 grams. The animals were housed in large steel cages and fed Purina Lab Chow *ad libitum*.

The rats were exposed to 1000 r of γ-radiation delivered from a 2000 Curie Cs^{137} source, filtered through one-half inch of Lucite. The dose rate was 80 r/min. In general, the rats were irradiated in a small, plastic screened cage containing two rats. Control and experimental animals were given exactly the same treatment with the exceptions of exposure to irradiation.

The procedures used to isolate mitochondria from spleen and liver have been described previously (Yost, Robson and Yost, 1967). The efficiency of oxidative phosphorylation was measured by use of the P:O ratio. For assay of the first step on the phosphorylating chain, glutamate was used as a substrate, and the medium was prepared according to the method of Hunter (1955). For assay of the second step, succinate was used as the substrate. With spleen mitochondria, the medium of Thomson (1964) was used, with the exception that the fluoride concentration was 26 μmoles per vessel. For assays of liver mitochondria, the medium of Yost and Robson (1959) was used with the modification that 36 μmoles phosphate and 65 μmoles of KF were used in each vessel. For assay at the terminal step, ascorbate was used to reduce cytochrome-*c*. Measurements were made using the method of Lehninger, Hassan and Sudduth (1964), with the exception that 26 μmoles of KF and 0.03 μmoles of cytochrome-*c* were used. In assays of the terminal step, it is important that cytochrome-*c* of very high purity be used; throughout, we always used Sigma 100%. Oxygen uptake was measured using a Warburg respirometer at 25° C. Readings were taken until 8–12 μatoms of oxygen had been consumed (usually 30 minutes), after which the reactions were stopped with TCA. Since we are interested only in the relative efficiency of the phosphorylating system, the oxygen consumption was held constant in each run and

Table I

A comparison of the uncoupling at each of the three steps in mitochondrial phosphorylation by irradiation

	I Glutamate			II Succinate			III Ascorbate		
	No. runs	Ave. P:O	% Inact.	No. runs	Ave. P:O	% Inact.	No. runs	Ave. P:O	% Inact.
Spleen									
Control	15	3.1	16**	12	2.0	25**	19	0.62	36**
Irradiated		2.6			1.5			0.40	
Liver									
Control	19	3.0	0	15	1.8	11*	25	0.94	16**
Irradiated		3.0			1.6			0.79	

* $P < 0.01$.
** $P < 0.001$.

TABLE II

The effect of varying swelling time and time of assay on the uncoupling at the terminal step of oxidative phosphorylation

	Time in hypotonic sucrose		
	5 m n.	10 min.	15 min.
No. runs	3	3	3
Control	0.96	1.00	1.00
γ	0.63	0.70	0.67
Inactivation	34%	33%	33%
	Post-irradiation time of assay		
	24 hrs.	72 hrs.	
No. runs	6	6	
Control	0.93	1.05	
γ	0.59	0.74	
Inactivation	36%	30%	

the time on the Warburg was allowed to vary. Phosphate determinations were made by the method of Lowry and Lopez (Glick, 1949). All phosphate determinations were made in duplicate. All assays were conducted 20 to 24 hours post-irradiation.

RESULTS

The data in Table I indicate that, taken individually, all three steps are significantly uncoupled by exposure to ionizing radiation when measured in mitochondria isolated from spleen. However, we were unable to observe any significant uncoupling of the primary step in liver mitochondria, under the conditions used in these experiments. As indicated by our previously reported data, the terminal step seems to be significantly more sensitive than the other two, and the initial step is the least sensitive.

Since Hall, Goldstein and Sonnenblick (1963) have reported the uncoupling of the primary step in liver at times lower than 24 hours post-irradiation, we felt that it would be advisable to determine whether we could observe uncoupling immediately post-irradiation. We chose 3 hours post-irradiation as a time that had given them a significant effect. Six runs were made. The controls averaged 3.1, varying between 2.9 and 3.3; whereas the experimentals averaged 3.2, varying between 3.0 and 3.5. Thus, it would appear that under our conditions, we cannot observe uncoupling in the primary step (in liver). In addition to these experiments, we made four runs assaying the uncoupling at the primary step in spleen at 3 hours post-irradiation. The controls averaged 2.8 (2.7–2.8); whereas the treated average was 2.7 (2.5–2.9). There is no significant difference between the two, and it seems unlikely that any significant uncoupling can be observed, this early, with spleen glutamate. These results are in agreement with those of Thomson, Nance and Bordener (1966).

To obtain adequate P:O ratios at the terminal step, it is necessary to "soak" the extracted mitochondria in hypotonic sucrose to increase the permeability to reduced cytochrome-*c*. We were concerned that this might accentuate the effect of the irradiation by slightly uncoupling the system. Consequently, studies were conducted on the effect of varying the time of treatment with hypotonic sucrose. The data in Table II indicate that the time in hypotonic sucrose does not have any appreciable effect on the P:O ratio, or on the level of inactivation. The normal time of soaking in hypotonic sucrose is 5 minutes (in no case longer than 7 minutes), and thus, whatever variations there are in the data cannot be attributed to the hypotonic treatment. In addition, Table II presents data showing that the effect of the irradiation on the uncoupling of the terminal step is relatively long-lasting as has been previously demonstrated for the uncoupling at the second step (Yost, Glickman and Beck, 1964).

Discussion

The data presented in this paper indicate that the majority of uncoupling of oxidative phosphorylation by ionizing radiation can be attributed to uncoupling at the terminal step. Obviously, it is difficult to assume that any particular value has special validity. Only the relationship of the values is important. Thus, if we arbitrarily pick the terminal step, we can set the expected values of inactivation for the other two steps relative to that empirically-derived value. If all the inactivation were coming from the terminal step, one would expect (in the spleen) that the second step would show one-half of the inactivation observed with the terminal step alone, or 18%. Similarly, one would expect one-third of the inactivation in the primary step, or 12%. The difference between the "expected" 12% and the observed 16%, in the first step, does not seem sufficient to suggest that there is any uncoupling of the primary step itself. At the second step, however, there seems to be more inactivation than would be expected simply from uncoupling of the terminal step alone. This suggests that the second site itself is partially damaged. However, it must be clear that the damage is relatively slight, since the majority of the inactivation observed can be accounted for simply on the basis of the inactivation occurring at the terminal step.

Approximately the same results as those discussed above were obtained with liver mitochondria. One would expect, working from the terminal step backwards, values of 16%, 8% and 6%. Failure to observe any inactivation at the primary step is difficult to explain. However, it may be that with "expected" percentage inactivations that low, even larger numbers of runs than those done for this paper would be necessary to demonstrate inactivation. The relatively good agreement for the second step suggests that any uncoupling of liver mitochondrial phosphorylation by exposure to ionizing radiation resides in the terminal step alone.

Further comment should be made on the disagreement between the results obtained with liver mitochondria and those obtained by Hall, Goldstein and Sonnenblick (1963). As we have pointed out elsewhere (Yost, Robson and Yost, 1967), the values obtained in studies of the uncoupling of phosphorylation are subject to a number of experimental modifications. In fact, the values that we now achieve in this laboratory are much lower than those we initially obtained (for

example, compare Yost, Glickman and Beck, 1964). In addition to differences in techniques, it is probable that commercially obtained rats are becoming progressively more resistant to some of the abscopal effects of irradiation, not through any design of the breeders but through improvement in overall disease resistance, etc. The fact that we failed to observe inactivation of liver glutamate should not be taken as an indication that experiments by other workers are invalid. Rather, they should serve to point to the fact that comparisons can be made only within one set of data. Considering the possible sources of variation in P:O ratios, this is hardly a surprising conclusion.

In 1960, we put forward an hypothesis that the effects of irradiation on the uncoupling of oxidative phosphorylation were largely abscopal in nature (Benjamin and Yost, 1960). At that time, it was suggested that it might be to the advantage of an organism to accelerate its metabolism, in order to provide the necessary intermediates required for the restitution of damage. If such a mechanism were operating, one might expect that the terminal step would be the most sensitive, since uncoupling the terminal step achieves the release from "tight coupling" control with the least possibility of damage to the rest of the phosphorylating chain. Thus, the observation that the terminal step is the most sensitive to the effects of whole-body irradiation is consistent with the hypothesis that the observed uncoupling is merely part of a more generalized response to stress.

Summary

White male rats of Sprague-Dawley strain were exposed to 1000 r total-body γ-irradiation. Measurements of the uncoupling at each of the three phosphorylating sites in mitochondria isolated from liver and spleen were made. The results indicate that the terminal step (oxidation of reduced cytochrome-*c*) is the most sensitive of the three and that the great majority, if not all, of the observed uncoupling may result from damage to this step.

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